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Recombinant *Escherichia coli* Clones Expressing *Chlamydia trachomatis* Gene Products Attach to Human Endometrial Epithelial Cells

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To identify *Chlamydia trachomatis* genes involved in attachment to host cells, a chlamydial genomic library was screened on the basis of binding characteristics by two methods. In the whole-cell screen, individual recombinant *Escherichia coli* clones were assayed for adherence to eukaryotic cells. In the membrane-binding screen, each recombinant colony of *E. coli* was treated with $CHCl_3$ and assayed for binding to purified, 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS)-solubilized, ^{35}S-labeled eukaryotic membrane material. Initial screening with McCoy cells was refined by using HEC-1B cells, a human endometrial epithelial cell line, which discriminate among recombinants adhering to McCoy cells. Some recombinants demonstrate significantly greater adherence to HEC-1B cells than to McCoy cells and appear, by transmission electron microscopy, to associate with electron-dense areas of the epithelial cell plasma membrane, resembling coated pits. Recombinants positive by one or both screening methods were examined by Southern and Western (immunoblot) analyses, which revealed the presence of chlamydial sequences inserted in the plasmids and the expression of novel 18-, 28-, and ~82-kDa proteins, recognized by antichlamydial sera. Maxicell analysis of selected recombinants confirmed that the proteins of 28 and ~82 kDa, and perhaps of 18 kDa, are plasmid encoded. Antiserum generated against the recombinant ~82-kDa protein reacted in Western analysis with a similar-sized protein from *C. trachomatis* serovar E elementary bodies (EB) and reticulate bodies, serovar L2 EB, and *C. psittaci* EB. *E. coli* JM109(pPBW58) contains a 6.7-kb plasmid insert which encodes proteins of all three sizes. Under a number of different conditions in the whole-cell attachment assay—i.e., at 4°C, in Ca^{2+}- and Mg^{2+}-free medium, in the presence of trypsin or dextran sulfate, and with rabbit aortic endothelial cells—the binding specificity of JM109(pPBW58) parallels that of *C. trachomatis* EB. Finally, the adherence phenotype of *E. coli* JM109(pPBW58) correlates directly with the presence of the recombinant plasmid; the phenotype is lost concurrently with loss of the recombinant plasmid, and the phenotype is acquired concurrent with reintroduction of the recombinant plasmid into *E. coli* JM109. The role of the 18-, 28-, and ~82-kDa proteins in mediating attachment, whether they act in concert as a complex or individually, has yet to be determined.

In nature, the obligate intracellular bacterium *Chlamydia trachomatis* demonstrates selectivity in the host organism (humans) and the types of host cells infected. *C. trachomatis* serovars A, B, Ba, and C infect conjunctival epithelia; serovars D through K primarily infect urogenital epithelia, though conjunctival and fetal bronchial epithelia are also susceptible. The lymphogranuloma venereum biovar (*C. trachomatis* serovars L1, L2, and L3) initially infects the urogenital epithelial but then invades deeper tissue, demonstrating a marked lymphotrophism. Cell selectivity in vivo has a number of plausible explanations, which include restriction to cell types to which the nonmotile, infectious *C. trachomatis* elementary bodies (EB) are exposed, and restriction to cell types capable of supporting chlamydial growth (30). Selective adherence only to cells capable of supporting growth is more economical, and certainly, as the processes of attachment and entry are of vital importance, one might suppose that chlamydiae have evolved a strategy to ensure their entry into the appropriate host cell.

Adherence and entry of *Chlamydia* spp. have been studied by numerous investigators, and some findings suggest that macromolecules on the infectious EB and the host cell participate in chlamydial attachment. Exposure of EB to conditions and reagents which reduce adherence to tissue culture cells include (i) exposure to heat (60°C) for 3 min, (ii) periodate treatment, and (iii) acetic anhydride treatment (4, 14). Adherence of chlamydiae is also reduced by exposing the tissue culture cells to periodate and trypsin; the effect of the latter is reversible over time except in the presence of cycloheximide, which inhibits eukaryotic protein synthesis (4, 14). These data suggest that the EB and the host cell have both surface carbohydrate and proteinaceous moieties, perhaps glycoproteins, which are involved in chlamydial attachment.

Several questions remain unanswered. (i) Do the treatments mentioned above affect some global surface property, such as charge or hydrophobicity, or are certain macromolecules, i.e., a chlamydial adhesin(s) and eukaryote receptor(s), involved in specific interactions that mediate attachment? (ii) Is chlamydial adherence a one-step process or is it a multistep process in which electrostatic interactions might mediate initial attachment but are followed by more specific interactions (49)?

A role for electrostatic interactions is suggested by findings that the polycation DEAE-dextran improves the infec-

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tivity of some serovars of *C. trachomatis* and that Ca^{2+} or Mg^{2+} is necessary for maximal adherence (14, 25). Since both the eukaryotic cell surface and chlamydiae carry a net negative charge, a formidable electrostatic barrier must be overcome prior to attachment (39, 47). Indeed, in an elegant study by Su et al. (44), the major outer membrane protein (MOMP) is proposed to promote localized, nonspecific electrostatic and hydrophobic interactions with the host cell. They demonstrated that monoclonal antibodies to MOMP surface-exposed epitopes in variable domains II and IV block EB attachment to hamster kidney cells (43, 44). The heat-sensitive loss of adherence of EB is correlated to a heat-triggered conformational change in an inaccessible, conserved, hydrophobic nonapeptide in the variable domain IV region of MOMP.

Data which support a specific adhesion step include evidence that (i) attachment of chlamydiae to HeLa or McCoy cells is saturable at 4°C and different serovars of *C. trachomatis* competitively inhibit adherence of another serovar (48) and (ii) chlamydiae can be internalized by the receptor-mediated endocytosis pathway (15, 16, 36, 40). Investigators have proposed that several chlamydial proteins are candidates for adhesins. For example, a polypeptide of 27 to 32 kDa (the size depending on the serovar) was shown to bind to isolated, radioiodinated HeLa cell membranes following denaturation with sodium dodecyl sulfate (SDS) (10, 51). Most recently, Swanson and Kuo (45) have discovered lectin-binding activity in chlamydial polypeptides of 18 and 27 to 32 kDa; lectin binding is abolished when the chlamydial proteins are treated with periodate. Joseph and Bose (19) have proposed that another protein, a 38-kDa "cytadhesin," is involved in chlamydial adherence. Of all the proteins solubilized in *n*-octyl-β-D-glucopyranoside extracts of whole EB, including the MOMP, 18-, and ~32-kDa proteins, only the 38-kDa protein was shown to bind specifically to glutaraldehyde-fixed HeLa cells at 4°C and not to bind to the plastic culture vessel. Both heating and trypsin treatment of the extract interfered with binding of the 38-kDa protein to host cells and resulted in a loss of competitive inhibition in the attachment of EB to host cells.

This study adopts a functional approach for identification of chlamydial adherence determinants, to either substantiate previous work or discover as yet unrecognized chlamydial adhesins. The 1,600 recombinant *E. coli* clones obtained from a *C. trachomatis* (serovar E) genomic library (pUC19) were screened individually by two assays. (i) Each recombinant was carefully examined for adherence to McCoy and subsequently to HEC-1B cell monolayers to determine whether expressed proteins of chlamydial origin could confer adherence to the normally nonadherent strain of *E. coli*. (ii) In the event that chlamydial proteins expressed in the recombinant could not be properly exported to and folded on the surface of *E. coli*, chloroform-exposed colonies of each recombinant were assayed for eukaryotic membrane-binding activity by incubation with ^{35}S-labeled eukaryotic membrane. Several recombinants that were positive by one or both methods are indeed producing novel proteins of chlamydial origin.

MATERIALS AND METHODS

Bacterial strains and culture methods. *C. trachomatis* E/UW-5/CX (obtained from C. C. Kuo and S. P. Wang, University of Washington) was grown in McCoy cells and purified, and the titer was determined as described previously (33). Reticulate bodies (RB) were purified by an adaptation of the endosome purification procedure of Matsumoto (31) followed by standard Renografin density centrifugation. The HEC-1B cell line used in screening and functional assays was propagated from a human endometrial adenocarcinoma (26). McCoy and HEC-1B cells were cultured in Eagle's minimum essential medium (MEM) with 10% heat-inactivated bovine calf serum. *E. coli* JM109 [*recA1 endA1 gyrA96 thi hsdR17* [r_K^- m_K^+] *relA1 supE44* λ^- Δ(*lac-proAB*) (F^- *traD36 proAB lacI*^{q}ZΔM15)] (53), JM109 (pUC19), and recombinants were grown at 37°C in Luria broth (LB) or on Luria plates with ampicillin (200 μg/ml) for plasmid-containing strains. Mid-log-phase bacteria were frozen in LB with 20% glycerol to generate freezer stock cultures.

Chlamydial DNA and plasmid preparation. Approximately 0.5×10^{10} to 5×10^{10} purified EB were used to isolate chlamydial genomic DNA as described previously (22). Generally, the purified genomic DNA fragments were greater than 50 kb in length, as determined by electrophoresis in a 0.3% agarose gel. pUC19 (53) and recombinant plasmids were isolated from *E. coli* by the alkaline lysis method of Birnboim and Doly (2) and CsCl gradient centrifugation.

Creation of a *C. trachomatis* genomic library. The pUC19 vector was chosen because (i) a complete genomic library could be generated with <2,000 recombinants and (ii) pUC vectors were successfully used to isolate chlamydial genes encoding outer membrane proteins and an enzyme which were stably expressed and functional, respectively, in *E. coli* (5, 34). A partial *Sau*3AI digest of *C. trachomatis* serovar E DNA was size fractionated on a 0.7% agarose gel. Fragments of 5 to 12 kb in length were electroeluted and ligated into pUC19, which had been digested with *Bam*HI and then dephosphorylated with calf alkaline phosphatase. Strain JM109 was made competent for transformation by the rubidium chloride method of Hanahan (13). Aliquots of the ligation reaction mixes were incubated with competent JM109 for 30 min on ice, then heated for 2 min at 42°C, and incubated for 90 min at 37°C. The bacteria were plated on LB plates containing ampicillin (100 μg/ml) and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) (20 μg/ml) and incubated for 16 h at 37°C. Recombinant colonies were selected on the basis of insertional inactivation of the alpha fragment of β-galactosidase on the plasmids, as determined by using the lactose analog X-gal. The vector, pUC19, did not require induction of the *lac* promoter (*lacZp*) by IPTG (lactose analog; isopropyl-1-thio-D-galactoside) to distinguish an active alpha fragment in the presence of X-gal. Apparently, the *lac* repressor does not completely stop transcription from *lacZp* on a high-copy-number plasmid such as pUC19. The chlamydial genomic library consisted of a total of 1,600 white colonies (β-galactosidase negative), which has a >99% probability of being complete if the chlamydial genome size is 1,450 kb (8). Each recombinant was inoculated into a well of a 96-well microtiter plate containing LB and ampicillin and incubated overnight without agitation in a wet box at 37°C; glycerol was added to 25% before the entire microtiter plate was frozen.

Whole-cell screen. Our whole-cell screening technique is a modification of the technique used successfully by Isberg and Falkow (17) to identify the *Yersinia pseudotuberculosis* invasin gene. The recombinant *E. coli* clones which constitute the chlamydial library were assayed individually for adherence to eukaryotic cells. All recombinant JM109 and control strains were inoculated directly from freezer stocks into LB containing ampicillin (500 μg/ml) and incubated with

agitation at 37°C until mid-log phase, as measured by optical density (A_{660} of ≤0.500). The bacteria were pelleted in a microcentrifuge tube, and each strain was resuspended to 10^8 bacteria per ml in Eagle's MEM containing 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) and ampicillin (500 μg/ml) but without fetal calf serum. For a multiplicity of infection of 50:1, 0.1 ml of each bacterial suspension was added to a subconfluent monolayer of approximately 2×10^5 eukaryotic cells in a 24-well culture plate. The cultures were incubated at 35°C for 1.5 to 2 h and shaken by hand every 15 min, in accordance with the procedure used in our laboratory for chlamydial infections (16, 33). After the attachment period, the monolayers were washed 5 to 10 times with sterile Hanks' balanced salts solution (HBSS), fixed with methanol for 2 min, and stained with Giemsa. The number of adherent bacteria per cell on 50 randomly chosen eukaryotic cells was determined by light microscopy with a 40× objective. Recombinants were scored as positive if an average of ≥5 bacteria attached per eukaryotic cell and none adhered to the tissue culture vessel. Both the negative control, nonadherent JM109(pUC19), and the positive control, piliated adherent *E. coli* ORN 115 (courtesy of P. Orndorff [32]), were included whenever the screening assay was performed.

Invasion assay. Recombinants selected as positive by the initial whole-cell screen were tested for invasion of eukaryotic cells by the gentamicin protection assay (6). Following the 2-h attachment period and extensive washing of the monolayers, gentamicin was added to 50 μg/ml in HBSS and the cultures were incubated at 35°C for an additional 1 h. Then the gentamicin solution was removed from the monolayers, the monolayers were washed with HBSS, and the infected eukaryotic cells were detached from the microtiter well with 0.1% EDTA in HBSS (without Ca^{2+} or Mg^{2+}). The eukaryotic cells were lysed in 1% Triton X-100 in HBSS; the bacteria were pelleted in a microcentrifuge and then plated onto LB plus ampicillin (200 μg/ml). Each resulting colony should represent one surviving bacterium which presumably invaded the eukaryotic cells.

Suspicious that some of the survivors represented false positives, i.e., bacteria not killed by gentamicin by virtue of their protected location in between and underneath the eukaryotic cells, the gentamicin assay was modified to be performed on eukaryotic cells in suspension. The washed infected monolayers were detached from the plastic microtiter well by addition of 0.1% EDTA in HBSS (with agitation), pelleted at 500 × *g*, resuspended in HBSS with gentamicin (50 μg/ml), and incubated in suspension at 35°C for 1 h. The eukaryotic cells were pelleted again and lysed, and the bacteria were plated as above.

Membrane-binding screen. An alternative screening method was designed in case the chlamydial adherence determinants expressed in the recombinants might not be translocated to the outer membrane of *E. coli*. McCoy and HEC-1B cells radiolabeled with 0.5 mCi of Tran^{35}S-label per 150-cm^2 flask (ICN Biomedicals, Inc.) were subjected to hypotonic lysis (31), and the radiolabeled eukaryotic membranes were isolated on Percoll gradients (density, 1.05 g/ml). Following velocity centrifugation at 100,000 × *g* for 1 h at 4°C, the purified, radiolabeled membranes were suspended in phosphate-buffered saline containing 0.5% 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS) (28).

The recombinants and the negative control, *E. coli* JM109(pUC19), were inoculated onto nitrocellulose filters placed on LB-ampicillin plates and incubated at 37°C for 12 h. The filters were removed the next day, spotted with 5 μl of a crude preparation of EB (the positive control), and treated with $CHCl_3$ fumes for 15 min to permeabilize the bacterial membranes. The filters were rinsed twice at 37°C with gentle agitation with blot buffer (50 mM Tris, 15 mM NaCl, 1 mM $CaCl_2$, 10 mM $MgCl_2$) containing protease inhibitors (200 μM phenylmethylsulfonyl fluoride, 1 μM pepstatin, and 1 μM leupeptin), blocked with 5% nonfat dry milk in blot buffer for 1 h, and incubated for 1.5 to 2 h with 10^6 cpm of ^{35}S-labeled membrane (as determined by scintillation counting) in blot buffer containing 0.5% CHAPS (28). The filters were rinsed three times with blot buffer, air dried, covered with Saran Wrap, and placed on film for autoradiography (X-OMAT AR; Eastman Kodak). Treatment with fluors was not required for enhancement of the signal.

Agarose gel electrophoresis and Southern blots. Restriction enzyme digests, agarose gel electrophoresis, and Southern blots were performed as described by Maniatis et al. (29), except the plasmid probes were labeled with dUTP-biotin by the random primer method (7). After the standard hybridization procedure at 65°C (29), the biotinylated probes were detected by using a streptavidin-alkaline phosphatase conjugate (24).

Curing the plasmid. To determine whether loss of the adherent phenotype could be correlated with loss of the recombinant plasmid before further characterization and subcloning proceeded, the recombinant plasmid was cured from the host *E. coli* by transformation into the same strain of a second plasmid, pHSS6 (Kan^r [38]), incompatible with pUC19. The pHSS6 plasmid carries different selectable markers than pUC19; therefore, under only kanamycin selection, pHSS6 will supplant the Ap^r recombinant plasmid after many generations. pHSS6 was transformed into the recombinant strains (JM109) by electroporation (1). The new transformants were initially selected on LB plates supplemented with ampicillin (200 μg/ml) and kanamycin (50 μg/ml). Subsequently, several transformants were chosen, grown to lag phase in LB broth containing only kanamycin (50 μg/ml), and repeatedly subcultured into fresh LB-kanamycin. The recombinants [JM109(pUC19 + chlamydial DNA, pHSS6)] were subcultured by this method three times a day for 5 days. When the phenotype was reexamined, there were no detectable Ap^r colonies remaining (<0.01%), and these bacteria were tested for adherence in the whole-cell binding assay.

SDS-PAGE and maxicell analysis. Total protein concentrations were determined by the bicinchoninic acid microassay (Pierce). Samples were subsequently heated at 100°C for 10 min in 0.12 M Tris-HCl (pH 6.8)–2% (wt/vol) SDS–2% (vol/vol) β-mercaptoethanol–20% (vol/vol) glycerol–0.01% (wt/vol) bromophenol blue. Solubilized proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) with a 15% (wt/vol) polyacrylamide separating gel and a 4% stacking gel, essentially as described by Laemmli (27). Protein profiles were visualized by staining with Coomassie brilliant blue.

Plasmid-encoded polypeptides were radiolabeled by maxicell analysis by a modification (41) of the original method of Sancar et al. (37). Since strain JM109 was found to be sensitive to UV irradiation (57.6 J/mm^2), the initial recombinants were used for these studies. Briefly, recombinants and controls were incubated in M9 broth, supplemented with 0.5% casamino acids and 0.4% glucose, to an A_{660} of 0.350 to 0.500 and then UV irradiated. These cultures were incubated overnight in the presence of cycloserine (200 μg/ml) to eliminate any viable bacteria. The next day, the bacteria

were washed, starved for 60 min at 37°C in M9 medium without casamino acids, and subsequently incubated at 37°C with either 25 μCi of Tran^{35}S-label (ICN Biochemicals) or 25 μCi of ^{14}C-mixed amino acids (New England Nuclear) for 1 or 4 h, respectively, to label newly synthesized proteins. The maxicell products were resolved by SDS-PAGE. Following fluorographic enhancement with Enlightening (New England Nuclear) radiographic enhancer for 15 min, the gels were dried (except for 20% polyacrylamide gels) and examined by autoradiography with X-ray film (X-OMAT AR; Eastman Kodak).

Production of polyclonal antisera and Western immunoblot analysis. *C. trachomatis* serovar E antisera were generated in female New Zealand White rabbits weighing 7 to 7.5 lb (ca. 3 to 3.4 kg). A Renografin-purified preparation of serovar E EB served as one inoculum, and EB which had been freshly treated with 10 mM dithiothreitol (DTT) in 2× sucrose-phosphate-glutamine buffer (2SPG) for 60 min at 37°C were used as a separate inoculum. DTT reduction was used to expose subsurface components perhaps masked by the tightly disulfide-bonded EB envelope. On examination by Macchiavello stain, the DTT-treated EB appeared blue, like RB (12). Both preparations were suspended in 2× sucrose-phosphate-glutamine buffer to a final concentration of 10^9 EB per inoculum. The immunization schedules involved six initial intravenous injections over a 2-week period, followed by monthly subcutaneous boosts over a 6-month period. The polyclonal sera were collected approximately 10 days after each subcutaneous boost.

Following SDS-PAGE, protein was electrophoretically transferred to nitrocellulose (0.2-μm pore size; Schleicher & Schuell) as described by Towbin et al. (46). Prior to blotting, antichlamydial antisera were adsorbed five times against JM109(pUC19) whole cells to reduce background cross-reactive epitopes and subsequently diluted 1:200 in phosphate-buffered saline plus 0.05% Tween 20. An alkaline phosphatase-conjugated anti-rabbit immunoglobulin G (IgG; Sigma Chemical Co.) was used for Western detection as described by Blake et al. (3).

Monospecific, polyclonal antiserum against the ~82-kDa protein was generated in a female New Zealand White rabbit. Total *E. coli* JM109(pPBW58) protein was resolved by preparative 8% polyacrylamide SDS-PAGE and visualized by staining with Coomassie brilliant blue, and the ~82-kDa band was excised. Protein was eluted from the gel slices into 1 M NaCl with an electrophoretic concentrator (LKB 2014 Extraphor; Pharmacia-LKB), dialyzed extensively against 10 mM potassium phosphate buffer (pH 7.0, 4°C), lyophilized, and stored in 5% (wt/vol) sucrose at −70°C. The preparation contained no detectable level of lipopolysaccharide (LPS) as determined by the 2-keto-3-deoxyoctulosonic acid microassay of Karkhanis et al. (20) and was diluted in saline prior to injection. Two intravenous injections, each containing 51 μg of protein, were administered on days 1 and 5. Subsequent subcutaneous injections of 34 μg of protein, in Freund's incomplete adjuvant, were administered twice weekly beginning on day 15. The IgG fraction was purified from collected immune serum with a Rec-protein G affinity column (Zymed Laboratories, Inc.), adsorbed against *E. coli* JM109(pUC19), and diluted 1:1,000 for Western blot analysis as described above.

TEM. The procedures for preparation of samples for transmission electron microscopy (TEM), embedding in Epon-Araldite resin, and staining of the ultrathin sections were the same as described previously (52). The sections were examined on a Philips 201 electron microscope operating at 60 kV.

RESULTS

Whole-cell screen. A functional screening method was used to identify possible chlamydial adherence determinants from a genomic library by testing whether the presence of expressed chlamydial genes inserted into pUC19 could make a nonadherent *E. coli* strain adhere to eukaryotic cells. Examination of Giemsa-stained infected monolayers by light microscopy (Fig. 1) allowed us to pay particular attention to the pattern of attachment of the adherent bacteria. For any recombinant to be considered adherent, it had to be attached to the eukaryotic cells and not to the plastic surface of the culture vessel, and an average of ≥5 bacteria had to be attached per eukaryotic cell. These criteria were determined by assessing the attachment patterns of a positive control, piliated *E. coli* ORN 115 (Fig. 1A), and of the negative control, JM109(pUC19), which rarely if ever attached (Fig. 1B). Although attachment of the piliated *E. coli* is mediated by a different mechanism than that in the nonpiliated recombinants, the attachment pattern of a known adherent *E. coli* strain served to define the criteria for distinguishing true adherence from "nonspecific trapping."

Of the 1,600 recombinants screened individually on McCoy cells, 30 to 40 recombinants were initially recorded as positive. Following a second screening, 25 recombinants were selected for further screening on the human endometrial epithelial cell line HEC-1B. The level of adherence increased in nine of these recombinants to an average of 10 to 15 bacteria per HEC-1B cell (Fig. 1C and D). The adherent recombinants were notably concentrated at the leading lamellapodial edge and thin processes of eukaryotic cells (Fig. 1C). Often there were large clusters of adherent bacteria on a single eukaryotic cell. We believed that this might be a eukaryotic cell cycle phenomenon for two reasons. All inocula had been carefully examined prior to addition to the monolayers, and clumping of the bacteria was not observed. Second, although the bacteria were in log phase, preliminary experiments had demonstrated that no appreciable bacterial cell division occurred during the adherence period when serum was omitted from the medium. Examination by TEM (Fig. 2) demonstrated that some recombinant *E. coli* were attached to indented electron-dense regions of the polarized epithelial plasma membrane, which resemble coated pits (Fig. 2D), but none appeared to be wholely within the cytoplasm.

Do the adherent recombinants enter epithelial cells? A standard invasion assay with gentamicin is based on the premise that the antibiotic does not readily penetrate eukaryotic cells. Therefore, gentamicin-sensitive bacteria are protected only if they have invaded eukaryotic cells. Initial results from the gentamicin assay found survivors of all adherent recombinant strains tested, suggesting that all were capable of invading the epithelial cells. However, TEM examination demonstrated that the bacteria were located in protected, extracellular niches, in between and underneath epithelial cells (data not shown). When the invasion assay was modified by adding gentamicin to a suspension culture of the infected epithelial cells, there were no survivors. Thus, none of the adherent recombinants appeared to enter the host cell.

Membrane-binding screen. An alternative screening method was undertaken to expose putative chlamydial adhesins which may not have been properly assembled on the surface

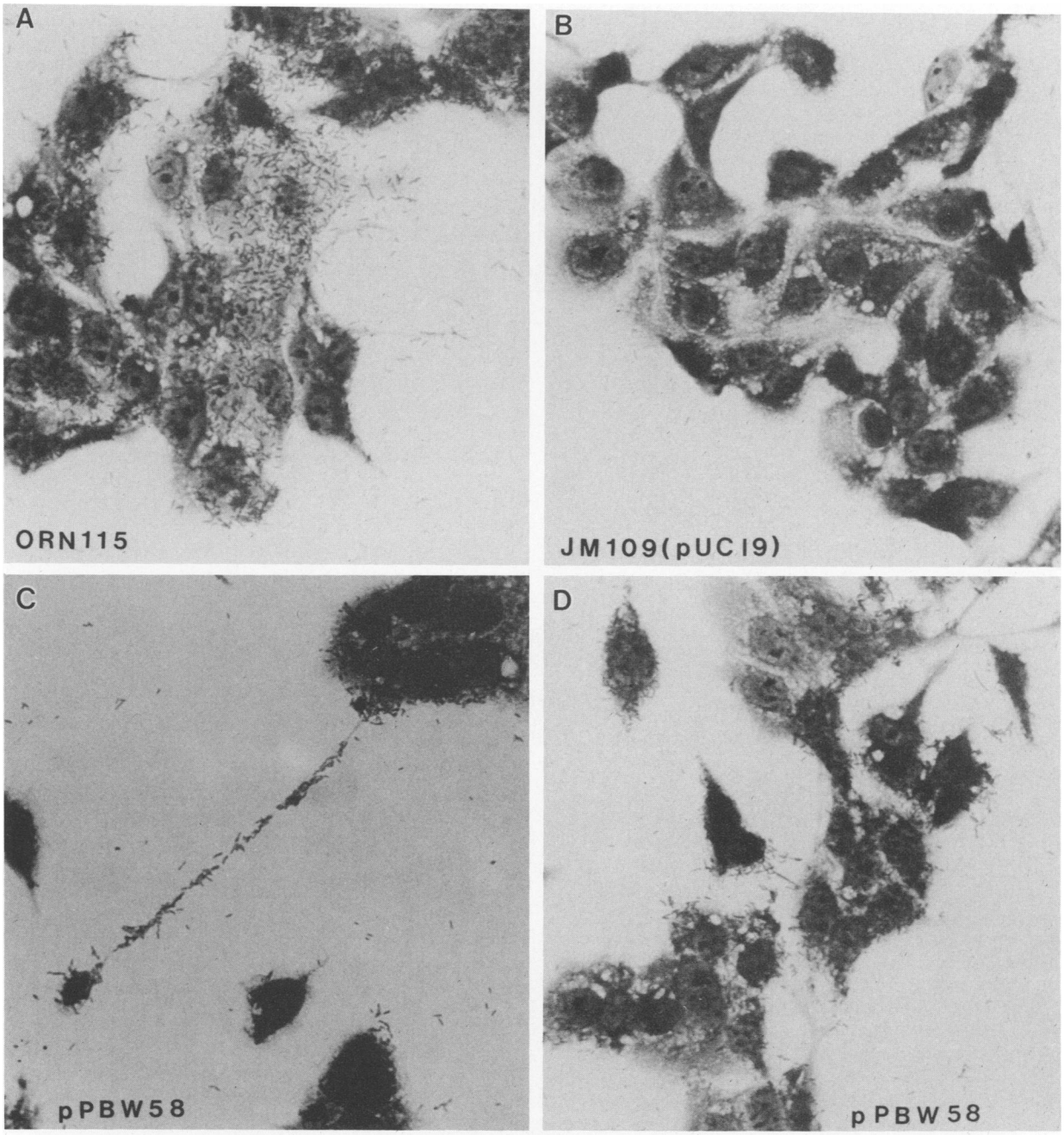

FIG. 1. Light photomicrographs of the whole-cell screen. (A) Positive control, piliated *E. coli* ORN 115; (B) negative control, JM109(pUC19); (C and D) adherent recombinants incubated with HEC-1B cells for 2 h at 37°C and stained with Giemsa. Magnification, ×400.

of *E. coli*, as required in the whole-cell screen. This approach involved permeabilization of the membrane of the recombinant *E. coli* with $CHCl_3$ and subsequent incubation with radiolabeled eukaryotic membranes. Crude preparations of McCoy cell membranes were solubilized in several different detergents: Triton X-100, octylglucoside, and CHAPS. Comparison of the signals from the positive control, *C. trachomatis* serovar E EB, and the negative control, JM109(pUC19), demonstrated that CHAPS gave the best signal-to-background ratio. In addition, the divalent cations Ca^{2+} and Mg^{2+} were necessary for maximum signal. The entire library was screened with intrinsically ^{35}S-labeled McCoy cell membranes and subsequently with ^{35}S-labeled HEC-1B membranes, and five recombinants were considered to be positive (Fig. 2F).

Only two recombinants were clearly positive by both screening methods, but this was not surprising since the location of expressed chlamydial adherence determinants in different *E. coli* compartments may affect binding function. The whole-cell screen appeared to be the more sensitive approach but was dependent on outer membrane localization and proper configuration of chlamydial gene products in *E.*

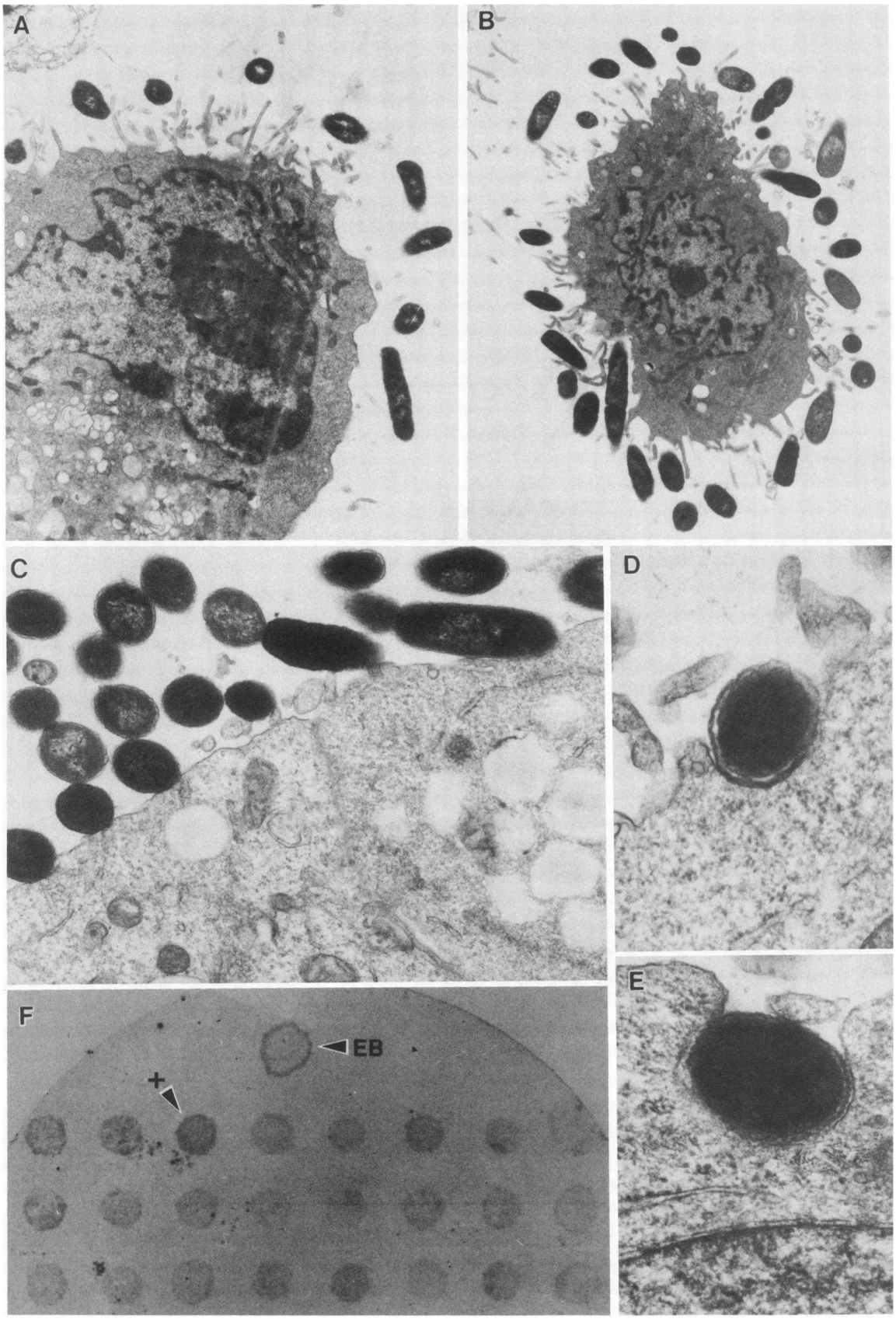
A
B
C
D
E
F
EB
+

FIG. 2. TEM photomicrographs of piliated *E. coli* ORN 115 (positive control) (A) and *E. coli* recombinants (B to E) attached to McCoy cells (A and B) and polarized human endometrial epithelial cells (C through E). A darkly staining invagination, suggestive of a coated pit, is visible in panel D. (F) Autoradiograph of a membrane-binding screen; the positive control, *C. trachomatis* (EB), and a positive recombinant (+) are indicated by arrows. The remainder of the spots are recombinants which are negative for this screen. Magnifications: (A) ×28,000; (B) ×18,000; (C) ×50,000; (D and E) ×150,000.

coli and could be compromised by nonspecific "stickiness" from overexpression of the proteins. Alternatively, it was possible that the chloroform permeabilization step in the membrane-binding assay altered the configuration of structurally sensitive domains necessary for a ligand-receptor interaction. Conversely, one or both methods may have selected for different characteristics than were originally sought, such as binding by charge phenomena versus specific adhesion. Recombinants positive by each assay were chosen for further examination.

Southern blot analysis of positive recombinants. Examination of selected recombinant plasmids by Southern blot verified that the positive recombinants contain chlamydial DNA sequences. Excision of the inserted sequences by *Pvu*II digestion followed by separation on an agarose gel revealed that the recombinant plasmids contain inserts varying from 2 to 6.7 kb in length. Southern blots of the gel with *Sau*3AI-digested total chlamydial DNA as a probe confirmed that the inserts are chlamydial in origin (data not shown). Southern hybridizations of the recombinant plasmids to a *Bam*HI digest of *C. trachomatis* serovar E DNA demonstrated that the chlamydial sequences on the plasmids are from different *Bam*HI fragments of the chlamydial genome; four examples are shown in Fig. 3. Each lane represents hybridization with a different recombinant plasmid. In each case, the number of chlamydial DNA fragments hybridizing to the recombinants is one greater than the number of *Bam*HI sites in the insert of the recombinant plasmid, suggesting that the chlamydial inserts in the recombinant plasmids are from a contiguous portion of the chlamydial genome. For example, the recombinant plasmid probe used in lane 1 does not have a *Bam*HI site in the chlamydial insert and thus hybridized to a single *Bam*HI fragment of chlamydial genomic DNA, whereas in lane 3, a single *Bam*HI site in the insert of the recombinant plasmid results in hybridization to two chlamydial fragments. Finally, Southern analyses of the inserts of various positive recombinants probed with inserts from other positive recombinants have not demonstrated any shared sequence, although all combinations have not been tested.

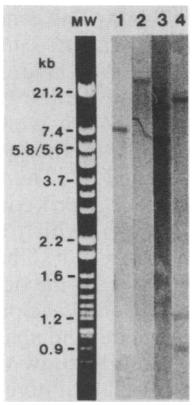

FIG. 3. Southern hybridization of biotinylated recombinant plasmids to *C. trachomatis* serovar E DNA. Lanes 1, 2, 3, and 4 are separate Southern blots of *Bam*HI-digested total *C. trachomatis* serovar E DNA, transferred from the same 1% agarose gel. Each lane of chlamydial genomic DNA was hybridized with a different recombinant plasmid. The number of chlamydial *Bam*HI fragments hybridizing to the recombinant plasmid probes is equal to the number of *Bam*HI sites in the plasmid plus 1, suggesting that each insert is a contiguous portion of the chlamydial genome. The lane designated MW is a photograph of the ethidium bromide-stained size markers, a mixture of *Eco*RI-digested lambda and a *Hin*cII and *Hin*dIII double digest of lambda.

Chlamydial proteins expressed by recombinants. A brief summary of certain characteristics of three recombinants examined to date is shown in Table 1 to illustrate the diversity within the library and, thus, the potential for examining what may be multiple attachment mechanisms. Twenty selected recombinants which exhibited varying degrees of attachment by one or both screening methods produced a combination of three novel proteins—~82, 28, and 18 kDa—shown by staining with Coomassie blue. Intensely stained bands of 28, 55, and approximately 82 kDa (±7 kDa) are visualized in *E. coli* JM109(pPBW58) (Fig. 4A, lane 3). It is presently difficult to designate an accurate molecular mass for the large polypeptide; it often migrates as a doublet and is susceptible to proteolytic breakdown. JM109(pPBW58) is reproducibly adherent by both screening assays, whereas JM109(pPBW57) (Fig. 4, lane 4) exhibits only minimal adherence in the whole-cell assay and produces only a 55-kDa protein. Unlike the 28- and ~82-kDa proteins, which are consistently produced at a high level of expression, the appearance of the 55-kDa protein is often difficult to reproduce. Continued examination revealed that production of this protein varies with growth conditions, such as temperature, nutrient source, phase of growth, and ampicillin concentration. For example, the first four lanes of Fig. 4A are from *E. coli* grown in the presence of 200 μg of ampicillin per ml; the last two lanes show *E. coli* grown in the presence of 500 μg of ampicillin per ml. The higher

TABLE 1. Summary of screening results and protein data for selected recombinants

Characteristic	Recombinant		
	pPBW56	pPBW57	pPBW58
Insert size (kb) of plasmid	5.8	2.2	6.7
Result in functional assays[a]			
Whole-cell screen	−	+	+++
Membrane-binding screen	+++	−	+
Protein(s) detected (kDa)			
Coomassie stain of SDS-PAGE gels	18 (weak)	55	28, 55, ~82
Western blot with anti-EB	−	−	18, ~82
Western blot with anti-DTT-EB	−	−	28, ~82
Maxicell analyses	35 (18?)	ND[b]	28, ~82 (18?)

[a] Results graded from strongly positive (+++) to negative (−).
[b] ND, not determined.

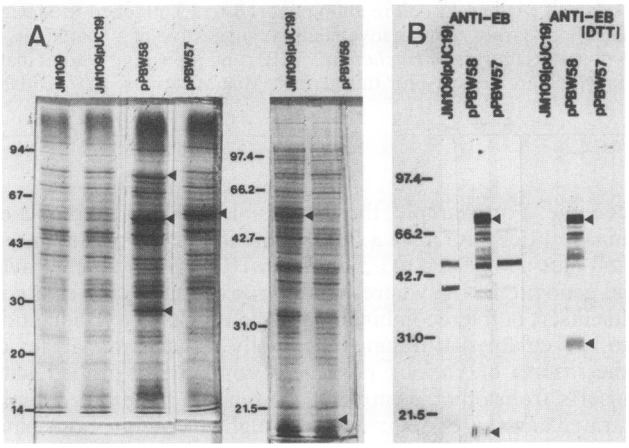

FIG. 4. Coomassie-stained SDS-polyacrylamide gels (A) and Western blots (B) of selected *E. coli* recombinants (JM109 carrying pPBW56, pPBW57, or pPBW58) compared with the *E. coli* host strains [JM109 and JM109(pUC19)]. Each lane was loaded with 25 to 30 μg of total bacterial protein. The migrational values of the novel protein bands (~82, 55, 28, and 18 kDa; arrowheads) were obtained by extrapolation from standard curves of low-range molecular weight markers (Bio-Rad and Pharmacia; correlation coefficients, ≥0.91). The numbers on the left indicate apparent molecular masses in kilodaltons. The polyclonal antisera used in the Western blots (B) were generated against *C. trachomatis* serovar E EB (anti-EB) or EB pretreated with 10 mM DTT (anti-EB-DTT).

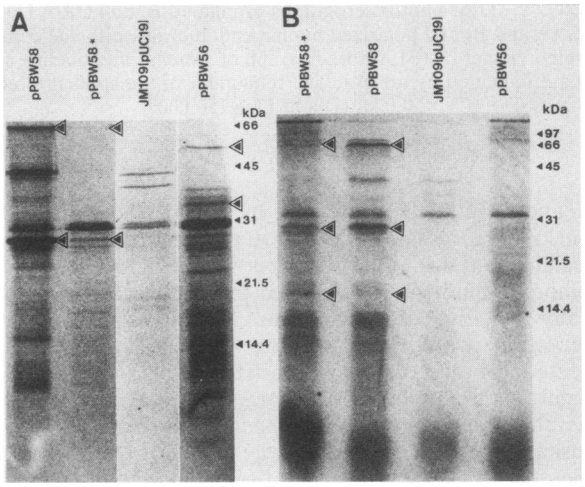

FIG. 5. Autoradiographs of maxicell analyses of recombinant plasmids. (A) ^{35}S-labeled proteins separated by 15% polyacrylamide SDS-PAGE. (B) ^{14}C-labeled proteins separated by 20% polyacrylamide SDS-PAGE. Positions of the protein standards are shown by the small arrows. Beta-lactamase is visible as a doublet at ~31 kDa in all the samples. Approximately 100,000 cpm of sample was loaded in each lane of both gels. The larger arrows point out polypeptides of interest. The ~82- and 28-kDa polypeptides are clearly visible in lane pPBW58 in both panels A and B, but the 55-kDa protein is not visible in either panel A or B. pPBW58* indicates recombinant *E. coli* clones which were transformed with pPBW58 but lost the adherence phenotype; these nonadherent recombinants produce less of the 28- and ~82-kDa proteins, as determined by maxicell analysis, than adherent recombinants containing pPBW58. pPBW56, as suggested by Western analysis, may encode an 18-kDa protein that is not visible in panel A and difficult to distinguish in panel B. However, a 35-kDa polypeptide is clearly visible when cells are labeled with [^{35}S]methionine.

concentration of ampicillin resulted in production of a 55-kDa protein in the host *E. coli* JM109(pUC19) (Fig. 4A, lane 5). Therefore, the 55-kDa protein appears to be an *E. coli* protein and is perhaps involved in stress response. We recognize that production of this protein may contribute to minimal levels of nonspecific attachment in the whole-cell screen; however, (i) JM109(pUC19) grown in the presence of ampicillin (500 μg/ml) is consistently nonadherent and (ii) JM109(pPBW58) is consistently adherent regardless of the presence or absence of abundant 55-kDa protein. Following exposure to chloroform, JM109(pPBW56) (Fig. 4A, lane 6) exhibits a striking affinity for eukaryotic membranes but is nonadherent in the whole-cell screen. The presence of an 18-kDa protein is consistently observed, although at a lower level of production relative to the 28-kDa and ~82-kDa proteins.

Selected recombinants were examined further for recognition of novel proteins by *C. trachomatis* serovar E polyclonal antisera (Fig. 4B). One antiserum was generated against EB, and the other antiserum was generated against EB pretreated with DTT (anti-DTT-EB antiserum) to expose subsurface components that may be masked by the tightly disulfide-bonded EB envelope. The ~82-kDa protein is strongly recognized by both antisera [*E. coli* JM109 (pPBW58), Fig. 4B, lanes 2 and 5), but the 28-kDa protein is detected only by the anti-DTT-EB antiserum (lane 5). As expected, the 55-kDa protein is not reactive against these preadsorbed antisera. *E. coli* JM109(pPBW58) also produces an 18-kDa protein which is recognized only by Western blotting with anti-EB serum (Fig. 4B, lane 2). The 18-kDa protein produced by *E. coli* JM109(pPBW56) is not detected by either antiserum, which suggests two separate 18-kDa proteins, one detectable by staining with Coomassie blue, and one detectable by antigenic recognition with chlamydial antiserum.

The antigenic recognition of the 18-, 28-, and ~82-kDa proteins by chlamydial antisera is encouraging, but to ascertain their origin from chlamydial gene sequences, maxicell analyses were conducted with *E. coli* JM109 recombinants carrying pPBW56 and pPBW58 (Fig. 5). With Tran^{35}S-label (70% ^{35}S-labeled methionine and 15% ^{35}S-labeled cysteine [ICN Biochemicals]; Fig. 5A), it is clearly evident that the 28- and ~82-kDa proteins originate from the chlamydial plasmid inserts (lanes 1 and 2). There are some additional bands, particularly a 35-kDa protein from *E. coli* JM109 (pPBW56) (Fig. 5A, lane 4), which are not detectable by Coomassie stain or Western blot. The 43-kDa band visualized in *E. coli* JM109(pPBW58) maxicells most likely represents a specific proteolytic cleavage product of the ~82-kDa protein; radioanalytic imaging and analysis (Ambis) of repetitive experiments consistently show that the amount of the 43-kDa protein coincides quantitatively with the amount of ~82-kDa protein produced.

Since some plasmid-encoded proteins may be Met and Cys deficient, the maxicell analysis was also conducted with a mixture of ^{14}C-L-amino acids (Fig. 5B). *E. coli* JM109 (pPBW58) (lanes 1 and 2) shows intense signals for the 28-kDa and ~82-kDa proteins, and a weak signal is detectable in the 18-kDa migrational range. Convincing evidence of plasmid origin for the 18-kDa protein(s) awaits sequence analyses, which are currently in progress.

To address the possibility that the expressed proteins might originate from gene fusions, more-specific antibody

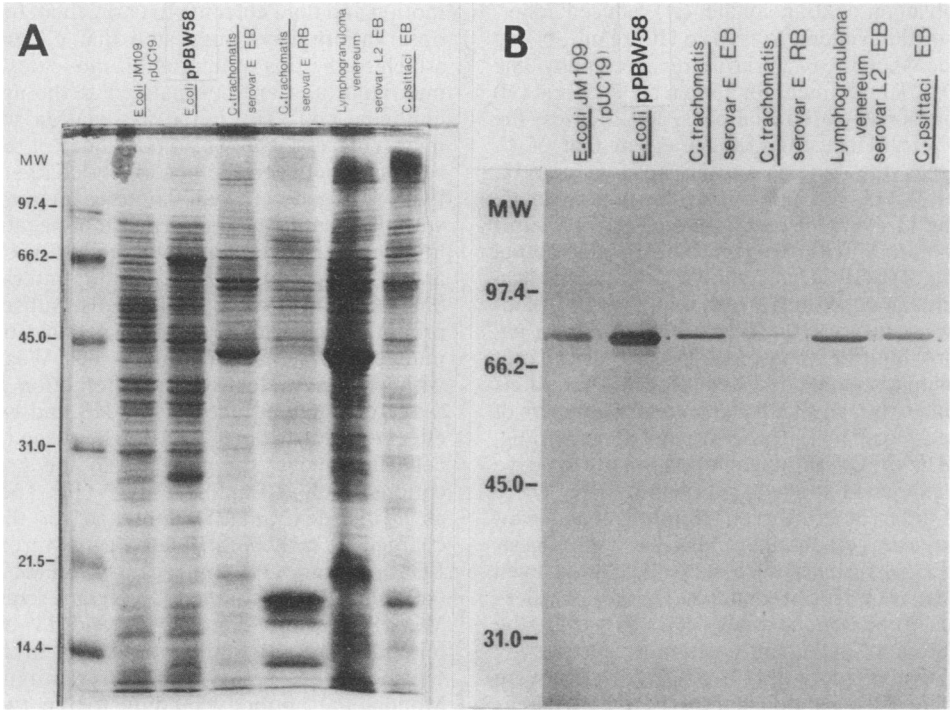

FIG. 6. Coomassie-stained SDS-polyacrylamide gel (A) and Western blot (B) of *E. coli* JM109(pUC19), recombinant *E. coli* JM109(pPBW58), *C. trachomatis* serovar E EB and RB, LGV serovar L2 EB, and *C. psittaci* Cal-10 EB. Each lane was loaded with 25 to 30 μg of total bacterial protein. The numbers on the left indicate molecular masses in kilodaltons. Polyclonal, monospecific antiserum against the ~82-kDa protein from JM109(pPBW58) was used for Western detection.

and nucleic acid probes are being generated. Particular emphasis is given to the newly described high-molecular-mass protein. Monospecific antiserum was generated against the ~82-kDa protein from JM109(pPBW58), eluted from SDS-PAGE gels. As shown in Fig. 6, the antiserum specifically recognizes a protein of similar size in highly purified *C. trachomatis* serovar E EB and RB (Fig. 6B, lanes 3 and 4), lymphogranuloma venerum serovar L2 (lane 5), and *C. psittaci* Cal-10 (lane 6).

Curing the plasmid. Although the whole-cell screen was conducted on the freshly generated recombinant library (three passages in broth culture), one early observation for *E. coli* JM109(pPBW58) was that a combination of multiple passages and freeze-thaw cycles resulted in decreased percentages of the adherent population and much lower expression of the 28- and ~82-kDa proteins [*E. coli* JM109 (pPBW58*), Fig. 5A and B]. Plasmid preparations do not indicate the presence of a contaminating plasmid. It is presently unclear whether this represents some type of transcriptional or translational regulation that reduces the level of expression of the chlamydial proteins, but a correlation between adherence to whole cells and the amount of recombinant protein produced is evident. The addition to log-phase *E. coli* JM109(pPBW58) cultures of 1 mM isopropylthiogalactoside, which induces the *lac* promoter, has no obvious effect on the level of expression of the 28- or ~82-kDa protein.

Two methods were used to link the adherent phenotype of recombinant JM109(pPBW58) with the presence of the plasmid pPBW58. In the first method, pPBW58 was cured from the recombinant, with concomitant loss of the adherent phenotype. The original recombinant was transformed with pHSS6, which is incompatible with pPBW58; selection for pHSS6 (Kn^r) resulted in loss of pPBW58 from the strain as well as loss of the ability to attach to epithelial cells. In the second method, the purified plasmid pPBW58 was reintroduced by electroporation into *E. coli* JM109; recombinant progeny had acquired the adherent phenotype and produced 28- and ~82-kDa proteins at levels comparable to those in the original recombinant.

Further analysis of recombinant *E. coli* JM109(pPBW58). Recombinant *E. coli* JM109(pPBW58) is an obvious candidate for additional studies for several reasons: (i) it is consistently positive in both binding assays, which correlates with the presence of the plasmid; (ii) it contains a large chlamydial insert (6.7 kb); and (iii) it produces ~82- and 28-kDa recombinant chlamydial proteins, and possibly one of 18 kDa, the relative amounts of which are reduced in nonadherent variant strains. *E. coli* JM109(pPBW56), which is only positive in the membrane-binding assay, also contains a large insert of 5.8 kb, but may be deficient in the ability to accommodate chlamydial proteins in a native, functional form. Restriction mapping to date shows little to no identity between pPBW56 and pPBW58, providing some support to the possibility of having cloned two separate genes encoding 18-kDa proteins.

Recognizing that the nonspecific stickiness of *E. coli* recombinants expressing chlamydial gene products could be a major factor in the results of the screening assays, the binding of recombinant JM109(pPBW58) to host cells was tested under various parameters known to affect the binding of *C. trachomatis* serovar E to the same host cells, including (i) reduced adherence at 4°C, (ii) reduced adherence in the absence of Ca^{2+} and Mg^{2+}, (iii) reduced adherence in the

presence of the polyanion dextran sulfate, (iv) reduced adherence on exposure of the bacteria to trypsin (10 μg/ml), (v) no change in adherence when the bacteria are rendered nonviable by sodium azide (0.02%) treatment for 15 min at 37°C, and (vi) adherence to rabbit aortic endothelial cells. In all cases, the binding specificity of JM109(pPBW58) paralleled that of *C. trachomatis*, whereas the binding of the positive control, piliated *E. coli* ORN 115, did not mimic the pattern of *C. trachomatis* binding in every instance. In addition, previous studies in our laboratory with primary human endometrial gland epithelial cells (HEGEC) have shown a differential susceptibility to chlamydial adherence which appears to be modulated by hormone exposure (30). Tissue obtained from patients in the proliferative phase (estrogen dominant), maintained in medium containing physiological concentrations of estrogen, was able to attach significantly greater numbers of chlamydiae than secretory-phase (progesterone dominant) tissue. Binding of JM109(pPBW58) to each hormone-responsive cell type has been examined in one experiment to date. There was essentially no difference in the numbers of recombinant bacteria adherent to estrogen-dominant HEGEC and progesterone-dominant HEGEC on a per-microscope-field basis, even though the cell density of estrogen-dominant HEGEC was less than that of progesterone-dominant HEGEC. However, the pattern of distribution of adherent bacteria was strikingly different. At least five or more JM109(pPBW58) cells were bound to susceptible estrogen-dominant HEGEC, whereas essentially only one bacterium was attached to susceptible progesterone-dominant cells.

JM109(pPBW58) in suspension and attached to HEC-1B cells was analyzed by indirect immunofluorescence with polyclonal anti-EB or anti-DTT-EB antibodies and then with fluorescein isothiocyanate-conjugated, second-affinity goat anti-rabbit IgG antiserum. There was no obvious specific labeling of the recombinant *E. coli* surface even though the bacterial outer membrane was perturbed by EDTA and exposed to lysozyme. It is possible that chlamydial proteins are masked by *E. coli* components, such as LPS, and/or that the interaction with the epithelial cell surface triggers exposure of functional epitopes. Alternatively, the antichlamydial antisera may not contain antibodies against surface-accessible epitopes. It should be noted that the polyclonal antichlamydial antisera used in these studies do not block adherence of infectious EB to target host cells. An initial experiment (data not shown) in which the inner and outer membrane of *E. coli* were separated by isopycnic sucrose gradient centrifugation (a modification of the method of Ito et al. [18]) indicates that the ~82-kDa protein is present in the outer membrane. The 28- and 18-kDa proteins appear to be associated with whole membrane prior to separation; however, the extent of this association, particularly in the presence of EDTA, is presently not clear and is under further investigation.

DISCUSSION

C. trachomatis is an adept bacterial parasite, as demonstrated by its high incidence in humans. In the United States, *C. trachomatis* genital infection is considered the most prevalent sexually transmitted disease, and in developing countries, *C. trachomatis* eye infection affects an estimated 500 million people. As an intracellular pathogen, infectious chlamydial EB must attach to and enter host cells to successfully initiate infection. How does a nonmotile, nonpiliated, negatively charged, metabolically inert EB adhere to host cells?

Lacking motility, chlamydiae must rely on Brownian motion and fluid currents to carry them to the host cell. TEM observations have suggested that *Chlamydia* spp. initially attach to the tips of microvilli, move down the length of the microvilli, and are internalized at the apical surface of the epithelial cell. The initial association with the tips of the microvilli is probably a consequence of reduced electrostatic repulsion between the negatively charged EB and the negatively charged yet small-diameter microvillus tip, compared with the relatively large expanse of negative charge found at the apical surface of the host cell (49). Originally, Hatch et al. (14) proposed that electrostatic interactions mediate the binding of chlamydiae to host cells. Subsequently, Ward (49) proposed that attractive nonspecific forces, hydrophobic interactions, and London-van der Waals forces compete with repulsive electrostatic interaction between the negatively charged surfaces of the EB and eukaryotic cell. The effect of electrostatic repulsion between the EB and the host cell surface may be reduced in the presence of divalent cations, such as Ca^{2+} and Mg^{2+} (14), and polycations, such as DEAE-dextran (25), more so for the more negatively charged *C. trachomatis* serovars A through K than for the LGV serovars (39). Indeed, in a recent study by Su et al. (44), negatively charged divergent sequences in exposed MOMP variable domains II and IV were suggested to function by electrostatic interactions in the initial binding of *C. trachomatis* serotype B to Syrian hamster kidney cells. Monoclonal antibody binding to the two domains significantly blocked attachment of chlamydiae to the host cells, presumably by interfering with the charge-dependent interactions (43, 44). According to the authors, a conformational change then occurred in the invariant nonapeptide sequences in variable domain IV, resulting in the exposure of a more specific, cryptic, hydrophobic binding site.

Since these binding studies were performed on Syrian hamster kidney cells rather than on the HeLa 229 cells in which the chlamydiae stocks were grown, there may be differences in the net negative charges as well as other surface properties between different epithelial cell types. An example of this possibility was noted in studies in our laboratory with primary human endometrial gland epithelial cells (HEGEC [30]), in which two populations of epithelial cells were distinguished by the fluorescence activated cell sorter; the one to which *C. trachomatis* serovar E bound possessed numerous microvilli and no obvious extracellular matrix; the second population, to which the chlamydiae did not bind, were devoid of microvilli and covered with a dense glycocalyx. Our failure to identify positive recombinants expressing MOMP by the functional screening assays might also be explained by the difficulty, reported by other investigators, of isolating recombinant *E. coli* expressing high levels of chlamydial MOMP (42).

Once electrostatic and hydrophobic interactions have drawn the chlamydiae close (~2 to 9 nm) to the target host cell, do other interactions involving additional chlamydial adherence determinants occur, such as specific intermolecular interactions (≤1 nm) between adhesins and receptors? Kihlstrom and Majeed (23) have recently reported that *C. trachomatis* EB first attach diffusely over the host cell but are mobilized into clusters before they are internalized. This observation is reminiscent of Fc receptor-mediated antibody capping on B-lymphocytes as well as receptor clustering into coated pits; the latter is part of the process of receptor-mediated endocytosis, which has been implicated in chlamydial uptake (15, 16, 36, 40). If movement of chlamydiae along the host cell membrane is caused by the directed movement of a eukaryotic receptor within the apical membrane, it follows that *C. trachomatis* EB may have a specific

adhesin(s). Although many of the chlamydial genes which had been isolated in *E. coli* have been identified by assaying for expression of the gene product with antichlamydial antisera, antiadhesin antibody has not been found. Thus, we used functional screening methods to identify putative adherence determinant gene products in recombinant *E. coli* clones.

These screening methods have identified several recombinants which bind eukaryotic membranes. However, recombinants that were adherent in the whole-cell screen could have their surface properties, such as charge or hydrophobicity, altered by the presence of foreign chlamydial proteins in their outer membrane. For instance, the recombinant *E. coli* which produces a chlamydialike LPS associates with HeLa cells two to five times more than the parental strain. Strains of *E. coli* lacking the LPS O antigen side chain demonstrate greater association with intestinal mucosa (35, 50). Likewise, recombinants positive for the membrane-binding blot may produce chlamydial proteins that have nonspecific membrane affinity. Chlamydial polypeptides of 18 and 27 to 32 kDa (the size of the latter depending on the serovar), after boiling in SDS, separation by SDS-PAGE, and electrophoretic transfer to nitrocellulose, were shown to bind to isolated, radioiodinated HeLa cell membranes. These proteins were purportedly involved in adherence (10, 21, 51).

Recently, the genes encoding the 18-kDa proteins have been cloned and sequenced. One gene product has predicted amino acid homology with eukaryotic histone H1, and the encoded protein has a calculated pI of 11.4 (11). The other gene appears to contain a fusion between two noncontiguous regions of genomic DNA, and the amino terminus of the fusion is a chlamydial homolog of the *E. coli* ribosomal protein L6 gene (9). Such proteins may have bound the iodinated membrane via nonspecific electrostatic interactions (11) or, alternatively, may have bound to nucleic acid still associated with the eukaryotic membranes. Though some of our positive recombinants are producing novel proteins of a similar size, the novel proteins do not appear to be homologous to the chlamydial histone H1-like protein, the ribosomal L6-like protein, or the 27- to 32-kDa protein. Monoclonal antiserum generated against the 27- to 32-kDa protein and polyclonal serum generated against purified 18-kDa (Hc1) protein (courtesy of T. Hackstadt) did not react with novel proteins produced by JM109(pPBW56) or JM109(pPBW58). Likewise, antiserum generated against the ribosomal L6 homolog did not react with novel proteins produced by our recombinants, nor did the chlamydial insert of pCT161/18 (both courtesy of W. Wenman) containing the ribosomal L6 gene homolog hybridize with our recombinant plasmids (pPBW56 and pPBW58) on a Southern blot. Furthermore, a polyclonal antiserum which reacts strongly with MOMP did not react with either JM109(pPBW56) or JM109 (pPBW58). It appears that we may have identified different chlamydial proteins which may function in chlamydial adherence. Yet the question still remains: are these novel chlamydial proteins synthesized in *E. coli* mediating a specific or nonspecific interaction with eukaryotic membranes? The increased signal and selectivity demonstrated by our positive recombinants when HEC-1B epithelial cells replaced McCoy cells in the screening assay suggest a certain specificity of the association. Also, the binding specificity of JM109(pPBW58) to HEC-1B cells and hormone-responsive primary HEGEC, which parallels that of *C. trachomatis*, is very suggestive of specificity.

The significance of the lack of internalization of the strongly adherent recombinants is not understood at this time. Assuming that these recombinants do express chlamydial adherence determinants, a number of explanations are possible, including (i) chlamydiae have several different adhesins, of which a subset triggers internalization; (ii) the mechanism of internalization of chlamydiae is size-restricted, excluding the larger *E. coli*; (iii) the "adhesin" density on the surface of *E. coli* is not great enough to trigger internalization; and/or (iv) possibly some enzymatic action occurring after adherence is required for internalization. Clearly, before any chlamydial gene products are described as adhesins, experiments other than those which establish some unspecified role in attachment are necessary to demonstrate that the proteins mediate specific interactions with epithelial cell receptors.

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